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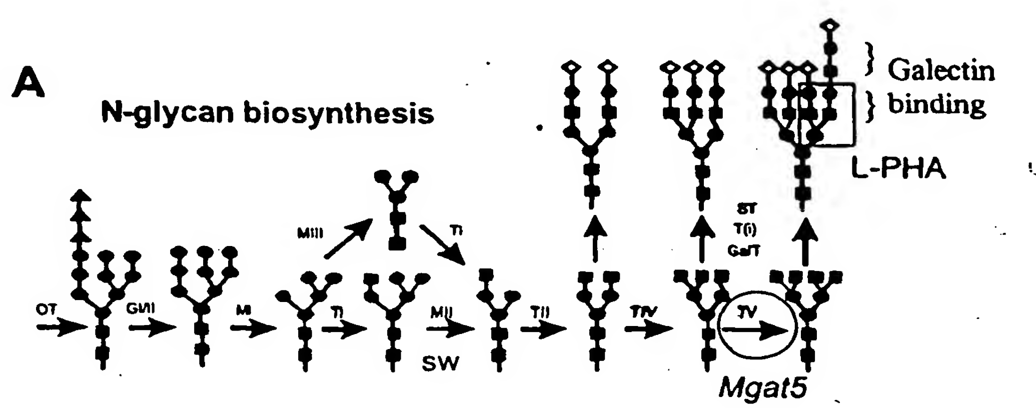
(54) Title: COMPOSITIONS AND METHODS FOR REGULATING RECEPTOR CLUSTERING



(57) Abstract: The invention relates to isolated complexes comprising one or more galectin associated with a Mgat5 modified glycan or polylactosamine modified glycan, and isolated lectin-Mgt5 modified glycan lattice comprising an array of mulitvalent interactions among lectins, Mgat5 modified glycans, polylactosamine modified glycans, and/or glycoproteins. Methods for evaluating a test compound for its ability to regulate receptor clustering through glycans on cell surfaces; and methods for regulating receptor clustering on cell surfaces comprising altering glycans on the cell surface associated with receptor clustering are also disclosed.

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These data indicate that a galectin-glycoprotein lattice strengthened by Mgat5-modified glycans restricts TCR recruitment to the site of antigen presentation.

In accordance with an aspect of the invention an isolated complex is provided comprising one or more lectin (e.g. a galectin) associated or interacting with a Mgat5 modified glycan or polylactosamine modified glycan that is associated with receptor clustering. The invention also provides a peptide derived from the binding domain of a lectin, preferably a galectin, that interacts with a Mgat5 modified glycan, or a polylactosamine modified glycan; and, an oligosaccharide derived from a Mgat5 modified glycan or a polylactosamine modified glycan that interacts with one or more lectin (e.g. a galectin). The invention also contemplates antibodies specific for these complexes, peptides, and oligosaccharides.

The invention also contemplates an isolated lectin-Mgat5 modified glycan lattice comprising an array of multivalent interactions among lectins, Mgat5 modified glycans, polylactosamine modified glycans, and/or glycoproteins that are associated with receptor clustering. The Mgt5 modified glycans and polylactosamine modified glycans are preferably part of glycoproteins of receptors including but not limited to TCR, growth factor receptors, and cytokine receptors.

Still further the invention provides a method for evaluating a test compound for its ability to regulate receptor clustering through glycans on cell surfaces (e.g. through Mgat 5 modified glycans and/or polylactosamine modified glycans) comprising assaying for alterations of the glycans in the presence of the test compound. Alterations of the glycans may increase or enhance, or inhibit or decrease receptor clustering thereby modifying signal transduction by the receptors.

In an aspect of the invention, a method is provided for evaluating a test compound for its ability to regulate receptor clustering through a lectin-Mgat5 modified glycan lattice, in particular a galectin-Mgat5 modified glycan lattice comprising determining the effect of the test compound on the lattice or a component thereof. A test compound may be a substance that interacts with a component of a lectin-Mgat5 modified glycan lattice. In particular, the substance may interact with a lectin (e.g. galectin), Mgat5 modified glycan, or polylactosamine modified glycan. The substance may be a molecule derived from a lectin (e.g. galectin), Mgat5 modified glycan, polylactosamine modified glycan, or lectin-Mgat5 modified glycan lattice; or, a substance which inhibits or enhances the interaction of a lectin (e.g. galectin) and a component of a lectin -Mgat5 modified glycan lattice (e.g. the interaction of a galectin and Mgat5 modified glycan and/or polylactosamine modified glycan).

In an embodiment, the method comprises (a) mixing a galectin-Mgat5 modified glycan lattice, or a galectin and one or more of a Mgat5 modified glycan and a polylactosamine modified glycan, and a test compound, under conditions which maintain the lattice or permit the formation of complexes between the galectin and one or more glycan; and (b) removing and/or detecting galectin-Mgat5 modified glycan lattice, complexes, galectin, Mgat5 modified glycan, or polylactosamine modified glycan.

The invention also encompasses the compounds identified using methods of the invention.

The invention also contemplates cell-based assays. In an aspect of the invention, a method is provided comprising (a) providing cells with receptors whereby clustering of the receptors is regulated

altered by modulating a glycosyltransferase enzyme (e.g. Mgat5) involved in the synthesis of the glycans.

In accordance with a particular aspect of the invention, a method is provided for treating or preventing a condition associated with decreased or increased receptor clustering (more particularly T cell receptor clustering), or a receptor clustering defect (more particularly a T cell receptor clustering defect), comprising modulating Mgat5 activity, the amount of Mgat5 modified glycans, polylactosamine modified glycans, or lectin-Mgat5 modified glycan lattice, and/or the amount of binding or interaction of one or more components of a lectin-MgatV modified glycan lattice (e.g. a galectin, a Mgat5 modified glycan, polylactosamine modified glycan, or glycoproteins).

The invention also contemplates compounds for regulating receptor clustering. The compounds may be capable of directly or indirectly modifiying glycans involved in receptor clustering. Such compounds may modulate the activity of an enzyme involved in the synthesis of the glycans (e.g. a glycosyltransferase such as Mgat5), the amount of the glycans, (e.g. the amount of Mgat5 modified glycans or polylactosamine glycans), and/or the amount of binding of the glycans with a substance that binds to the glycans thereby regulating receptor clustering (e.g. the binding or interaction of Mgat5 modified glycans and galectins). The invention also provides methods for assaying for such compounds. Compositions comprising such compounds are also within the scope of the invention.

In accordance with an aspect of the invention there is provided a method of, and products for, diagnosing and monitoring conditions characterized by an abnormality in clustering of a receptor comprising assaying for differential glycosylation of the receptor. Differential glycosylation may be assayed by determining the presence of Mgat5 modified glycans, polylactosamine modified glycans, lectin-Mgat5 modified glycan lattice, or an alteration or change in such glycans or lattice, compared to a control.

The invention relates to the control of glycan-lectin combinations (e.g. glacetin-polylactosamine modified glycan lattice) identified using the invention.

These and other aspects, features, and advantages of the present invention should be apparent to those skilled in the art from the following drawings and detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 Immune phenotype in Mgat5$^{-/-}$ mice (A) Schematic of the Golgi N-glycan biosynthesis pathway shows Mgat5 (TV) in the production of a tetra (2,4,2,6) antennary (numbers in brackets refer to the linkages of the antennae left to right). Abbreviations are oligosaccharyltransferase, OT; the α-glucosidases, GI, GII; the β-N-acetylglycosaminyltransferases, TI, TII, TIV, TV T(i); the α1,2mannosidases, MI, α1,3/6mannosidases MII, MIII; β1,4-galactosyltransferases, Gal-T; α-sialyltransferases, ST; SW, position of swainsonine block. The boxed structure Galβ1,4GlcNAcβ1,6(Galβ1,4GlcNAcβ1,2)Manα binds L-PHA. The galectin binding disaccharide N-acetyllactosamine (Galβ1,4GlcNAc) is present in all antennae, and units are marked with red brackets in polylactosamine. (B) Distribution of CD4+ and CD8+ cells in spleen and thymus by FACS analysis

0/10, 1/10 and 9/10, respectively. (D) *Mgat5*$^{+/+}$ T cells incubated with increasing concentrations of disaccharide (1/3 serial dilution from 2.4 mM) and stimulated for 1 min with anti-CD3ε antibody-coated beads were compared for phosphotyrosine. Arrows at the right indicate the positions of molecular mass markers. A longer exposure of the lower molecular weight portion of the blot is shown. (E) Galectin-3 detected by surface labeling with NHS-biotin on T cells. Below, association of galectin-3 with CD3ε and TCRα chain, and its disruption by Mgat5 deficiency and lactose is shown. (F) A model depicting restricted mobility of TCR by interaction with a galectin - glycoprotein network, which is stronger in Mgat5-expressing cells. (G) *LacZ* activity in untreated (white) and anti-CD3 and anti-CD28 stimulated (grey) T cells from *Mgat5*$^{-/-}$ mice. (H) L-PHA binding to *Mgat5*$^{+/+}$ T lymphocytes either untreated (white) or stimulated with anti-CD3 and anti-CD28 for 48h (grey).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional biochemistry, enzymology, molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See for example, Sambrook, Fritsch, & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M..J. Gait ed. 1984); Nucleic Acid Hybridization B.D. Hames & S.J. Higgins eds. (1985); Transcription and Translation B.D. Hames & S.J. Higgins eds (1984); Animal Cell Culture R.I. Freshney, ed. (1986); Immobilized Cells and enzymes IRL Press, (1986); and B. Perbal, A Practical Guide to Molecular Cloning (1984).

Complexes, Peptides, and Oligosaccharides

In accordance with an aspect of the invention an isolated complex is provided comprising one or more lectin associated or interacting with a Mgat5 modified glycan, or polylactosamine modified glycan that is associated with receptor clustering.

The term "isolated complex" refers to a complex substantially free of cellular material or culture medium when produced *in vitro*, or chemical reactants, or other chemicals when chemically synthesized.

"Lectin" refers to a molecule that interacts with, binds, or crosslinks carbohydrates. Preferably a lectin employed in the present invention interacts with, binds, or crosslinks Mgat5 modified glycans polylactosamine modified glycans, and/or glycoproteins. In an embodiment, the lectin is a galactose-binding protein, preferably a galectin.

"Galectin" refers to a member of the galectin family of beta-galactoside-binding proteins (see "Galectins: A Family of Animal beta-Galactoside-Binding Lectins" (1994) by S. H. Barondes, V. Castronovo, D. N. W. Cooper, R. D. Cummings, K. Drickamer, et al., In Cell 76, 597-598). Galectins includes lectins that bind beta-galactoside carbohydrate moieties in a thiol-dependent manner. (Reviewed in Hadari, Y. R. et al. (1998) J. Biol. Chem. 270:3447-3453.) Galectins are widely expressed and developmentally regulated. Galectins contain a characteristic carbohydrate recognition domain (CRD). The CRD is about 140 amino acids and contains several stretches of about 1-10 amino acids that are highly conserved among all galectins. Examples of galectins are galectin-1 through -10. In preferred

homology or complete homology. In an embodiment of the invention a glycostyltransferase, in particular Mgat5, is substantially homologous to a wild type enzyme. A sequence that is "substantially homologous" refers to a partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid. Inhibition of hybridization of a completely complementary sequence to the target sequence may be examined using a hybridization assay (e.g. Southern or northern blot, solution hybridization, etc.) under conditions of reduced stringency. A sequence that is substantially homologous or a hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of reduced stringency. However, conditions of reduced stringency can be such that non-specific binding is permitted, as reduced stringency conditions require that the binding of two sequences to one another be a specific (i.e., a selective) interaction. The absence of non-specific binding may be tested using a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% homology or identity). The substantially homologous sequence or probe will not hybridize to the second non-complementary target sequence in the absence of non-specific binding.

A sequence of an enzyme contemplated by the invention may have at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity. The phrases "percent identity" or "% identity" refer to the percentage comparison of two or more amino acid or nucleic acid sequences. Percent identity can be determined electronically using for example the MegAlign program (DNASTAR, Inc., Madison Wis.). The MegAlign program can create alignments between two or more sequences according to different methods, e.g., the Clustal method. (See, e.g., Higgins, D. G. and P. M. Sharp (1988) Gene 73:237-244.) Percent identity between nucleic acid sequences can also be determined by other methods known in the art, e.g., the Jotun Hein method. (See, e.g., Hein, J. (1990) Methods Enzymol. 183:626-645.) In addition, identity between sequences can be determined by other methods known in the art, e.g., by varying hybridization conditions.

"Mgat5 modified glycan" refers to a GlcNAcβ1,6Manα1,6-branched N-glycan structure. The glycans are produced by Mgat5 which catalyzes the addition of β1,6GlcNAc to N-glycan intermediates found on newly synthesized glycoproteins transiting the medial Golgi (15). The glycans are elongated in trans-Golgi to produce tri (2,2,6) and tetra (2,4,2,6) antennary N-glycans. A Mgat5 modified glycan may be substituted with for example polylactosamine (i.e. it may be a polylactosamine modified glycan). A Mgat5 modified glycan may be part of or covalently linked to a cell surface glycoprotein, including a glycoprotein of the T cell receptor complex.

"Polylactosamine modified glycan" refers to specific glycan structures comprising N-acetyllactosamine (Galβ1,4GlcNAc) and polymeric forms of N-acetyllactosamine, also known as poly N- acetyllactosamine or polylactosamine (6). Preferably the polylactosamine modified glycan is an Mgat5 modified glycan substituted with poly N-acetyllactosamine. A polylactosamine modified glycan may be part of or covalently linked to a cell surface glycoprotein, including a glycoprotein of the T cell receptor complex.

retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide, or enhancer or inhibitor of the invention.

The invention also contemplates an altered glycan of a cell surface glycoprotein associated with receptor clustering resulting from the inhibition of a glycosyltransferase involved in the synthesis of the glycan. In an embodiment the altered glycan is an altered Mgat5 modified glycan or an altered polylactosamine modified glycan. By way of example, an altered Mgat5 modified glycan has a deficiency of β1-6 branches, and an altered polylactosamine modified glycan has a deficiency of N-acetyllactosamine or polylactosamine. An altered Mgat5 modified glycan or altered polylactosamine modified glycan cannot substantially interact or associate with a lectin, preferably a galectin.

Mgat5 modified glycans, polylactosoamine modified glycans and altered glycans may be assayed using substances that bind to the glycans. The substances that bind to the glycans may be antibodies or lectins. For example, leukoagglutinin (L-PHA) is a tetravalent plant lectin that binds specifically to Mgat5 modified glycans.

The invention contemplates antibodies specific for the complexes, lattice, peptides, oligosaccharides, and altered glycans of the invention. Antibodies include intact monoclonal or polyclonal antibodies, and immunologically active fragments (e.g. a Fab, $(Fab)_2$ fragment, or Fab expression library fragments and epitope-binding fragments thereof), an antibody heavy chain, and antibody light chain, a genetically engineered single chain Fv molecule (Ladner et al, U.S. Pat. No. 4,946,778), humanized antibodies, or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

Antibodies specific for a Mgat5 modified glycan, polylactosamine modified glycan, complex, lattice, or an altered glycan may be produced in $Mgat5^{-/-}$ mice using conventional methods.

Antibodies specific for the complexes, lattice, peptides, oligosaccharides, and altered glycans of the invention may be used to detect the complexes, lattice, etc. in tissues and to determine their tissue distribution. *In vitro* and *in situ* detection methods using the antibodies of the invention may be used to assist in the prognostic and/or diagnostic evaluation of disorders mediated by or involving receptor clustering, more particularly T cell receptor mediated disorders. Antibodies specific for the complexes, lattice, etc. of the invention may also be used therapeutically to modulate receptor clustering, more particularly T cell receptor clustering (i.e. T cell activation).

Evaluating Compounds that Regulate Receptor Clustering

The invention provides a method for evaluating a test compound for its ability to effect or regulate receptor clustering through glycans on cell surfaces (e.g. glycans of the receptor such as Mgat5 modified glycans or polylactosamine modified glycans). Changes to glycans on cell surfaces may increase or decrease receptor clustering thereby modifying signal transduction by the receptors.

amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), oligosaccharides, antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, $F(ab)_2$, and Fab expression library fragments, and epitope-binding fragments thereof)], and small organic or inorganic molecules. The substance or compound may be an endogenous physiological compound or it may be a natural or synthetic compound.

The invention particularly contemplates a method for evaluating a compound for its ability to modulate the biological activity of a complex or lattice of the invention, by assaying for an agonist or antagonist (i.e. enhancer or inhibitor) of the binding or interaction of molecules in the complex or lattice. The basic method for evaluating if a compound is an agonist or antagonist of the binding of molecules in a complex or lattice of the invention, is to prepare a reaction mixture containing the molecules and the substance under conditions which permit the formation of complexes or a lattice, in the presence of a test compound. The test compound may be initially added to the mixture, or may be added subsequent to the addition of molecules. Control reaction mixtures without the test compound or with a placebo are also prepared. The formation of complexes or a lattice is detected and the formation of complexes or a lattice in the control reaction but not in the reaction mixture indicates that the test compound interferes with the interaction of the molecules. The reactions may be carried out in the liquid phase or the molecules, or test compound may be immobilized as described herein.

It will be understood that the agonists and antagonists i.e. inhibitors and enhancers that can be assayed using the methods of the invention may act on one or more of the binding sites on the interacting molecules in the complex or lattice including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of molecules in a complex or lattice of the invention. Thus, the invention may be used to assay for a compound that competes for the same binding site of a molecule in a complex or lattice of the invention.

In an embodiment, the method comprises mixing a lectin-Mgat5 modified glycan lattice or a component thereof (e.g. lectin such as a galectin, a Mgat5 modified glycan, polylactosamine modified glycan), and a test compound under conditions which maintain the lattice or permit the formation of complexes between the lectin and one or more of the Mgat5 modified glycan, and polylactosamine modified glycan, and removing and/or detecting lectin-Mgat5 modified glycan lattice, complexes, lectin, Mgat5 modified glycan, or polylactosamine modified glycan. The invention also encompasses the compounds identified using this method of the invention.

Substances which modulate the activity of a complex or lattice of the invention can be identified based on their ability to bind to a molecule in a complex or lattice of the invention. Therefore, the invention also provides methods for identifying novel substances which bind molecules in a complex or lattice of the invention. Substances identified using the methods of the invention may be isolated, cloned and sequenced using conventional techniques.

conditions which permit the formation of a lectin-Mgat5 modified glycan lattice, complexes between a lectin and one or more glycan of the lattice, and/or receptor clustering; (c) detecting a lectin-Mgat5 modified glycan lattice, complexes, lectin, Mgat5 modified glycan, polylacotsamine modified glycan, alterations to the lattice, complexes, lectin, Mgat5 modified glycan, or polylactosamine modified glycan, or detecting receptor clustering; and (d) comparing to a control to determine if the test compound potentially regulates receptor clustering.

In another aspect of the invention, a method is provided comprising (a) providing cells with receptors whereby clustering of the receptors is regulated through a lectin-Mgat5 modified glycan lattice or component thereof (e.g. lectin, Mgat5 modified glycans, and polylactosamine modified glycans); (b) mixing the cells, lectin, a test compound, and a ligand for the receptor that induces receptor clustering, under conditions which permit receptor clustering; (c) detecting receptor clustering; and (d) comparing to a control to determine if the test compound potentially regulates receptor clustering. The lattice on the cell surface may regulate the threshold cooperativity and dynamic range of ligand dependent responses.

In an embodiment of the invention a method is provided which comprises;

(a) mixing cells with T cell receptors comprising Mgat5 modified glycans or polylactosamine modified glycans, one or more galectin, and a test compound under conditions suitable for producing a galectin-Mgat5 modified glycan lattice;
(b) assaying for a galectin-Mgat5 modified glycan lattice; and
(c) comparing to a control in the absence of the test compound to determine if the test compound has the potential to regulate receptor clustering.

In an embodiment of the invention a method is provided which comprises;

(a) mixing cells with T cell receptors and Mgat5 modified glycans and/or polylactosamine modified glycans on their surface, one or more galectin, and a test compound under conditions suitable for producing receptor clustering of the T cell receptors;
(b) assaying for T cell receptor clustering or T cell signaling or activation;
(c) comparing to a control to determine if the test compound has the potential for regulating receptor clustering.

In a further embodiment of the invention, a method is providing for evaluating a test compound for its potential to regulate receptor clustering comprising:

(a) mixing cells with T cell receptors and Mgat5 modified glycans or polylactosamine modified glycans on their surface, one or more galectin, and a test compound under conditions suitable for producing a galectin-Mgat5 modified glycan lattice;
(b) assaying for Mgat5 modified glycans, polylactosamine modified glycans, galectin, or a galectin-Mgat5 modified glycan lattice, or alterations to the glycans or lattice;
(c) comparing to a control where an alteration to a Mgat5 modified glycan, polylactosamine modified glycan, galectin, or a galectin-Mgat5 modified glycan lattice, indicates that the test compound has potential to regulate receptor clustering.

Mgat5, under conditions whereby the Mgat5 is capable of transferring the sugar donor to the acceptor substrate to produce a sugar donor-acceptor substrate complex, and determining the effect of the substance by assaying for sugar donor-acceptor substrate complexes, unreacted Mgat5, unreacted sugar nucleotide donor or unreacted acceptor substrate.

Suitable acceptor substrates include a saccharide, oligosaccharides, polysaccharides, glycopeptides, glycoproteins, or glycolipids which are either synthetic with linkers at the reducing end or naturally occurring structures, for example, asialo-agalacto-fetuin glycopeptide. The sugar donor may be a nucleotide sugar, dolichol-phosphate-sugar or dolichol-pyrophosphate-oligosaccharide, for example, uridine diphospho-N-acetylglucosamine (UDP-GlcNAc), or derivatives or analogs thereof.

The Mgat5 may be obtained from commercial sources; it may be purified from immortalized cell lines such as small cell lung cancer cells such as QG (Gu, J. et al. J. Biochem. 113, 111-116, 1993); or prepared by expression of the gene encoding Mgat5 in host cells.

The acceptor substrate or sugar donor may be labeled with a detectable substance, and the interaction of the enzyme with the acceptor and sugar donor will give rise to a detectable change. The detectable change may be colorimetric, photometric, radiometric, potentiometric, etc. The activity of Mgat5 may also be determined using methods based on HPLC (Koenderman et al., FEBS Lett. 222:42, 1987) or methods employed synthetic oligosaccharide acceptors attached to hydrophobic aglycones (Palcic et al Glycoconjugate 5:49, 1988; and Pierce et al, Biochem. Biophys. Res. Comm. 146: 679, 1987).

The Mgat5 is reacted with the acceptor substrate and sugar donor at a pH and temperature and in the presence of a metal cofactor, usually a divalent cation like manganese, effective for the enzyme to transfer the sugar donor to the acceptor substrate, and where one of the components is labeled, to produce a detectable change. It is preferred to use a buffer with the acceptor substrate and sugar donor to maintain the pH within the pH range effective for the proteins. The buffer, acceptor substrate, and sugar donor may be used as an assay composition. Other compounds such as EDTA and detergents may be added to the assay composition.

Substances that inhibit or stimulate Mgat5 activity may also be assayed by treating immortalized cells that express Mgat5 with a substance suspected of inhibiting or stimulating Mgat5, and comparing the morphology of the cells with the morphology of the cells in the absence of the substance and/or with immortalized cells that do not express Mgat5.

Still further, a substance that inhibits or stimulates Mgat5 activity may also be identified by treating a cell that expresses Mgat5 with a substance that is suspected of affecting Mgat5 activity, and assaying for Mgat5-modified glycans or polylactosamine modified glycans on the surface of the cell. Mgat5-modified glycans and polylactosamine modified glycans can be measured using methods described herein and known in the art. For example, cells expressing Mgat5-modified glycans may be treated with a substance suspected of inhibiting or stimulating Mgat5-modified glycans. A lectin such as L-PHA is then added and the amount of binding can be compared to control cells which have not been treated with the substance and/or which do not express Mgat5-modified glycans.

Examples of reporter genes are genes encoding a protein such as β-galactosidase (e.g. lac Z), chloramphenicol, acetyl-transferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This makes it possible to visualize and assay for expression of recombinant molecules to determine the effect of a substance on expression of the glycosyltransferase (e.g. Mgat5) gene.

Mammalian cells suitable for carrying out the present invention include any malignant cells, for example, COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), and 293 (ATCC No. 1573). Suitable expression vectors for directing expression in mammalian cells generally include a promoter. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR.

Protocols for the transfection of mammalian cells are well known in the art and include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et al., Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989).

An agent that modulates Mgat5 activity, the amount of Mgat5 modified glycans, or the amount of binding of MgatV modified glycans and lectins (e.g. galectins) may comprise a complex of a lectin (e.g. galectin) associated with a Mgat5 modified glycan and/or a polylactosamine modified glycan, or a lectin-Mgat5 modified glycan lattice; a peptide derived from the binding domain of a lectin (e.g. galectin) that interacts with Mgat5 modified glycan or polylactosamine modified glycan; and/or an oligosaccharide derived from the Mgat5 modified glycan that interacts with a lectin (e.g. galectin).

The reagents suitable for applying the methods of the invention to evaluate substances and compounds that modulate receptor clustering, more particularly T cell receptor clustering, may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

Regulating Receptor Clustering

The invention provides a method for regulating receptor clustering on cell surfaces comprising altering glycans on the cell surface associated with receptor clustering, in particular altering receptor glycosylation. Preferably the receptors are those that comprise Mgat5 modified glycans or polylactosamine modified glycans. Examples of receptors include receptors that stimulate immune reactions (e.g. T cell receptors, Ig receptors, B cell receptors, NK receptors), the HER family of transmembrane receptor tyrosine kinases [e.g. epidermal growth factor (EGF) receptor also known as HER1 or Erb1, HER2 (neu, Erb2), HER3 (Erb3), and HER4 (Erb4)], cadherin receptors (e.g. E-cadherin and N-cadherin), interleukin (IL) receptors including IL-2 receptor, TNFγ receptor, and integrins.

Glycosylation may be altered by modulating one or more glycosyltransferase enzyme involved in the synthesis of glycans involved in receptor clustering, in particular N-glycans and N-glycan

Glycans can be altered or modified by modulating a glycosyltransferase enzyme involved in the synthesis of the glycans.

In accordance with a particular aspect of the invention, a method is provided for treating or preventing a condition associated with decreased or increased receptor clustering, more particularly T cell receptor clustering, comprising modulating Mgat5 activity, the amount of Mgat5 modified glycans, polylactosamine modified glycans, and/or lectin-Mgat5 modified glycan lattice, and/or the amount of binding or interaction of one or more MgatV modified glycans, polylactosamine modified glycans and lectins e.g. galectins.

A receptor clustering defect may be involved in conditions such as autoimmune diseases or proliferative disorders such as cancer.

A condition associated with increased T cell receptor clustering may include a T cell mediated autoimmune disease such as insulin-dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, myasthenia gravis, systemic lupus erythematosus, autoimmune hemolytic anemia, glomerulonephritis, enhanced delayed type hypersensitivity, allergic conditions, hypersensitivity, and autoimmune encephalomyelitis. Conversely, T cell recognition of cancers and immune therapy of cancer is limited by weak stimulation of T cells by tumor cells. The present invention may also be used to treat cancers susceptible to immune modulation.

In an aspect the invention contemplates a method for treating or preventing an autoimmune disease in a subject comprising reducing T cell receptor clustering in the subject. T cell receptor clustering is reduced by increasing the amount of Mgat5 modified glycans, polylactosamine modified glycans, and/or lectin-Mgat5 modified glycan lattice on the surface of T cells of the subject. In an embodiment, the method comprises up regulating or increasing the amount of Mgat5.

In an aspect the invention contemplates a method for treating or preventing cancer in a subject comprising increasing T cell receptor clustering in the subject. T cell receptor clustering is increased by decreasing the amount of Mgat5 modified glycans, polylactosamine modified glycans, and/or lectin-Mgat5 modified glycan lattice on the surface of T cells of the subject. In an embodiment, the method comprises down regulating or decreasing the amount of Mgat5.

In an embodiment of the invention, a method is provided for treating or preventing a condition associated with a growth factor receptor, in particular epidermal growth factor receptor, comprising regulating clustering or oligomerization (e.g. dimerization) of the growth factor receptor by altering glycosylation of the receptor, modulating Mgat5 activity, the amount of MgatV modified glycans, polylactosamine modified glycans, and/or the binding of MgatV modified glycans or polylactosamine modified glycans and lectins for the glycans. Inhibition of growth factor receptor clustering may be useful in treating conditions involving aberrant growth factors including but not limited to cancers such as solid human cancers, NSCL, breast cancer, head and neck cancer, gastric cancer, prostate cancer, bladder cancer, ovarian cancer, colorectal cancer, gliobastomas, and renal cell carcinoma.

One or more agents may be used to regulate receptor clustering. In particular, one or more agents may be used to modulate glycosyltransferase activity, more particularly Mgat5 activity, the

Agents, compounds, and substances described herein or identified using a method of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions that may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances or compounds in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The activity of a pharmaceutical composition, an agent, compound, or substance described herein or identified using a method described herein may be confirmed in animal experimental model systems.

In accordance with an aspect of the invention there is provided a method of, and products for, diagnosing and monitoring conditions characterized by an abonormality in receptor clustering comprising assaying for differential glycosylation of the receptor. Differential glycosylation may be assayed by determining the presence of Mgat5 modified glycans, polylactosamine modified glycans, lectin-Mgat5 modified glycan lattice, or an alteration or change in such glycans or lattice, compared to a control.

In an embodiment, a method of, and products for, diagnosing and monitoring conditions characterized by an abnormality or defect of receptor clustering involving the interaction of a galectin and Mgat5 modified glycan or polylactosamine modified glycan is provided comprising determining the

poly-L-lysine coated cover slips. The cells were fixed with 10% formalin, stained with 2 μg/ml fluorescein isothiocyanate (FITC) labeled anti-TCRα/β antibody (Pharmigen), solubilized with 0.2% Triton X-100, labelled with rhodamine-phalloidin and Hoechst, and then visualized by deconvolution microscopy. For disaccharide competition, wild type T cells were incubated for 20 min with 0, 0.01, 0.03, 0.09, 0.27, 0.8, and 2.4 mM of disaccharide prior to exposure to anti-CD3ε antibody beads. To measure TCR internalization, purified splenic T cells stimulated with either 0.1 $\mu g/ml^{-1}$ anti-CD3 antibody or with 10 ng/ml PMA for varying lengths of time were harvested and stained with FITC-anti-TCRα/β. PMA concentrations were not limiting as 10, 50 and 100 ng/ml produced similar internalization and cell activation results. To measure actin reorganization, purified splenic T cells, stimulated with 0.1 $\mu g/ml^{-1}$ anti-CD3 for varying lengths of time, were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS and stained with rhodamine-phalloidin and mean fluorescence intensity (MFI) was determined by FACS.

TCR signaling: T cells ($1x10^6$) and anti-CD3ε antibody coated beads ($5x10^6$ at 0.4 μg/ml antibody) in 100μl RPMI 1640 were pelleted, incubated at 37°C for various times, then solublized with ice cold 50 mM Tris pH 7.2, 300 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail (Boeringer Mannheim) and 2 mM orthovanadate. Zap-70 was immunoprecipitated by incubating whole cell lysates with rabbit polyclonal anti-Zap-70 agarose conjugate (Santa Cruz) overnight at 4° C, followed by one wash with lysis buffer and 3 washes with PBS. Western blotting were done with whole cell lysates or immunoprecipitates separated on SDS-PAGE gels under reducing conditions, transferred electrophoretically onto PVDF membranes and immunoblotted with antibodies to Akt/PKB (NEB), phospho-Akt/PKB (NEB), phosphotyrosine (clone 4G10, Upstate Biotechnology), Zap 70 (clone Zap-70-6F7, Zymed), TCRα (polyclonal, Santa cruz) and rabbit anti-galectin-3 (Dr. A Raz, University of Michigan). Cell surface proteins were biotinylated using sulfosuccininmidobiotin (NHS-biotin) for 30 min, PBS pH 8.0. Cells were lysed and labeled protein was captured on streptoavidin-agarose beads. To cross-link surface proteins on purified naïve T cells, the homobifunctional cross-linker dithio-bis(sulfosuccinimydylpropionate (DTSSP) was used at 0.1 mg/ml with 10^6 cell/ml in PBS pH 8.0 for 10 min at 20°C. T cells were preincubated for 20 min with or without 2 mM lactose, and reacted with DTSSP in the presence of the same. Aliquots of cell lysate were immunoprecipitated with rabbit anti-galectin-3 antibody or non-immune rabbit serum (NS), separated on reducing- SDS-PAGE and Western blotted for CD3ε and TCRα chain. The band above CD3ε is cross-reactivity of secondary antibody with light-chain.

To measure Ca^{++} mobilization, purified T cells were loaded with 10 μM AM ester of Fluo-3 (Molecular Probes) washed and stimulated with 10 μg/ml of anti-CD3ε antibody at 37°C. Emission at 525 nm was taken using a spectrofluorimeter with excitation at 488 nm. Data is plotted as a fraction of the Ca^{++} mobilized by addition of 2 $\mu g/ml^{-1}$ of ionomycin. *LacZ* activity in *Mgat5*$^{-/-}$ T cells was detected by loading cells with flourescin-di-β-D-galactopyranoside (FDG) (Molecular Probes) at 10°C, and

Alterations in cell surface TCR complex levels and intracellular signaling potential of T cells were examined and ruled-out as possible causes of the *Mgat5*$^{-/-}$ hypersensitivity. The Mgat5 deficiency did not significantly alter cell surface expression of CD3, CD4, CD8, TCRα/β, CD28 or CTLA-4 glycoproteins in resting T cells (Figure 1B,C and data not shown). Intracellular signaling potential in *Mgat5*$^{-/-}$ T cells is normal, as treatment with the phorbol ester PMA and the Ca^{++} ionophore ionomycin stimulated T cells equally well from mice of both genotypes (Figure 2D).

The relationship between cell surface *Mgat5*-modified glycans and T cell activation was examined. Leukoagglutinin (L-PHA) is a tetravalent plant lectin and commonly used T cell mitogen that binds specifically to Mgat5-modified glycans. *Mgat5*$^{-/-}$ T cells were completely unresponsive to L-PHA, confirming that Mgat5-modified glycans are required for stimulation by this lectin (Figure 2E). L-PHA reactive N-glycans are also present on B cells, but L-PHA is not a B cell mitogen. Furthermore, B cell responses to anti-IgM antibody, LPS and IL-4 plus anti-CD40 antibody were similar for cells from *Mgat5*$^{-/-}$ and *Mgat5*$^{+/+}$ mice (Figure 2F and data not shown). In T cells, L-PHA induces signaling common to TCR engagement, including phosphorylation of CD3ζ, Ca^{++} mobilization, PKC-γ and Ras/mitogen-activated protein kinase (Mapk) activation (18; 19). The TCRα/β chains have 7 N-glycans in total, and some are branched complex-type structures with L-PHA reactivity (20; 21). These data indicate that Mgat5-modified glycans are present on glycoproteins of the TCR complex and required for L -PHA mitogenesis.

When bound to major histocompatibility complex (MHC)/peptide, TCRs cluster with an inherent affinity greater than unligated TCR and the stability of these clusters is critical for intracellular signaling (22). However, the density of TCRs measured at the site of T cell–APC (antigen-presenting cell) contact is only marginally increased relative to the remaining cell surface, leaving the majority of the TCRs unengaged by MHC/peptide (4). It is possible that ligand induced TCR clustering in the plane of the membrane may be increased in the absence of Mgat5-modified glycans, thus lowering *Mgat5*$^{-/-}$ T-cell activation thresholds. To visualize TCR reorganization in response to an antigen-presenting surface, polystyrene beads were coated with anti-CD3ε antibody and incubated with purified *ex* vivo T cells. After 10 minutes of contact, TCRs in *Mgat5*$^{-/-}$ cells was markedly more concentrated at the bead surface compared to *Mgat5*$^{+/+}$ cells (Figure 3A,B). TCRs on wild type cells could not be induced to cluster to the same extent as Mgat5$^{-/-}$ cells even with longer incubations (20 min) or using anti-CD3ε plus anti-CD28 coated beads (data not shown). Actin microfilaments were more concentrated at the bead contact site in *Mgat5*$^{-/-}$ cells, and overlapped more extensively with TCR in the merged images compared to *Mgat5*$^{+/+}$ T cells (Figure 3A,B). TCRs are internalized following productive TCR clustering (1), and this was significantly greater in *Mgat5*$^{-/-}$ compared to *Mgat5*$^{+/+}$ cells (Figure 3C, solid lines). Intracellular signaling mediated by PMA treatment induces TCR internalization but at similar rates in *Mgat5*$^{-/-}$ and *Mgat5*$^{+/+}$ cells (Figure 3C, dotted lines). Microfilament re-organization was more rapid in *Mgat5* deficient T cells following soluble anti-CD3ε antibody stimulation (Figure 3D). Akt/protein kinase B (PKB) phosphorylation is dependent upon phosphoinositide 3-OH kinase activity, which stimulates Rac/CDC42 GTPases and actin filament re-organization (23). Phosphorylated Akt/PKB displayed a

in vitro (9; 25), antagonizes TCR signaling (26), and when injected into mice, it suppresses the pathology of EAE (27).

The gene replacement vector used to produce the Mgat5-deficient mice contained the reporter gene LacZ replacing the first exon, which was expressed with the same tissue-specificity as *Mgat5* transcript (16). Both LacZ expression and cell-surface Mgat5-modified glycans in *Mgat5*$^{+/-}$ T cells, respectively, increased 48h after stimulation demonstrating regulation of Mgat5 by transcriptional means (Figure 4G,H). This suggests that Mgat5 enzyme activity and glycan production are limiting in resting T cells, and with stimulation, increases in Mgat5-modified glycans and galectins may dampen TCR sensitivity to antigen. Negative feedback by Mgat5-modified glycans on TCR sensitivity is delayed as it requires Mgat5 gene expression, which is dependent on T cell activation status, and only indirectly on antigen concentrations. This form of slow-negative regulation governed by steady-state activity of the system is a key feature of robust and adaptive biochemical pathways (28) and Mgat5-modified glycans may contribute this feature to T cell regulation.

Viola *et al* have estimated that sustained clustering of ~8000 TCRs is required for T cell activation, but other molecular interactions clearly alter this threshold. With CD28 co-stimulation, only ~1500 TCRs are required (2). Co-signaling through CD28 decreases the extent of TCR clustering needed for activation predominantly by recruiting protein kinase-enriched GM1 ganglioside rafts to the site of TCR engagement, thereby amplifying signaling (3; 5). Here it is shown that Mgat5 deficiency increases the number of TCRs recruited to the antigen-presenting surface, thereby reducing the requirement for CD28 co-receptor engagement. This may lead to T cell activation in the absence of CD28 co-signaling, failure of anergy and loss of immune tolerance. *CD28*$^{-/-}$ mice are resistant to induction of EAE by low dose MBP, while *Mgat5*$^{-/-}$ are hypersensitive, but both mutants develop clinical signs of EAE comparable to wild type littermates with high doses of MBP (29). In this regard, CD28 and Mgat5 appear to be opposing regulators of T cell activation thresholds, and susceptibility to autoimmune disease. In summary, Mgat5-dependent glycosylation limits agonist-induced TCR clustering by sequestering receptors in a cell surface galectin –glycoprotein lattice. However, the glycosylation deficiency in *Mgat5*$^{-/-}$ mice affects other pathways and cells types that may also contribute to the observed autoimmunity. Indeed, Mgat5-modified glycans also reduce clusters of fibronectin receptors causing accelerated focal adhesion turnover in fibroblasts and tumor cells; a functionality that may affect leukocyte motility (16). Finally, glycosylation of Notch receptor by Fringe, a fucose-specific β1,3GlcNAc-transferase provides another example of regulation by differential receptor glycosylation (30). In a broad context, the results described herein suggest a general mechanism for the regulation of receptor clustering through differential glycosylation and interaction with cell surface lectins.

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We Claim:

1. An isolated complex comprising one or more galectin interacting with a Mgat5 modified glycan or polylactosamine modified glycan that is associated with receptor clustering.
2. An isolated lectin-Mgat5 modified glycan lattice comprising an array of multivalent interactions among lectins, Mgat5 modified glycans, polylactosamine modified glycans, and/or glycoproteins that are associated with receptor clustering.
3. An isolated lectin-Mgat5 modified glycan lattice as claimed in claim 2 wherein the Mgt5 modified glycans and polylactosamine modified glycans are part of glycoproteins of receptors, preferably T cell receptors.
4. A method for evaluating a test compound for its ability to regulate receptor clustering through glycans on cell surfaces comprising assaying for alterations of the glycans in the presence of the test compound.
5. A method for evaluating a compound for its ability to regulate receptor clustering through a lectin-Mgat5 modified glycan lattice comprising (a) mixing a lectin-Mgat5 modified glycan lattice, or a lectin and one or more of a Mgat5 modified glycan and polylactosamine modified glycan, and a test compound, under conditions which maintain the lattice or permit the formation of complexes between the lectin and one or more of the Mgat5 modified glycan and polylactosamine modified glycan; and (b) removing and/or detecting lectin-Mgat5 modified glycan lattice, complexes, lectin, Mgat5 modified glycan, or polylactosamine modified glycan
6. A method as claimed in claim 5 wherein the lectin-Mgat5 modified glycan lattice is a galectin-Mgat5 modified glycan lattice and the lectin is a galectin.
7. A cell based assay for evaluating a test compound for its ability to regulate receptor clustering through a lectin-Mgat5 modified glycan lattice or component thereof comprising (a) providing cells with receptors whereby clustering of the receptors is regulated through a lectin-Mgat5 modified glycan lattice or one or more of a lectin, Mgat5 modified glycan, or polylactosamine glycan; (b) mixing the cells, lectin, and a test compound under conditions which permit the formation of a lectin-Mgat5 modified glycan lattice, complexes between the lectin and one or more glycan, and/or receptor clustering; (c) detecting a lectin-Mgat5 modified glycan lattice, complexes, lectin, Mgat5 modified glycan, polylactosamine modified glycan, or alterations to the lattice, complexes, lectin, Mgat5 modified glycan, or polylactosamine modified glycan and/or receptor clustering; and (d) comparing to a control to determine if the test compound alters the lectin-Mgat5 modified glycan lattice or a component thereof and potentially regulates receptor clustering.
8. A compound identified using a method of claim 4, 5, 6, or 7.
9. A method for regulating receptor clustering on cell surfaces comprising altering glycans on the cell surface associated with receptor clustering.

22. A method as claimed in claim 21 wherein the glycans are altered by modulating a glycosyltransferase enzyme involved in the synthesis of the glycans, preferably Mgat5.

23. A method for treating or preventing a condition associated with decreased or increased receptor clustering, or a receptor clustering defect, comprising modulating Mgat5 activity, the amount of Mgat5 modified glycans, polylactosamine modified glycans, or lectin-Mgat5 modified glycan lattice, and/or the amount of binding or interaction of one or more components of a lectin-MgatV modified glycan lattice.

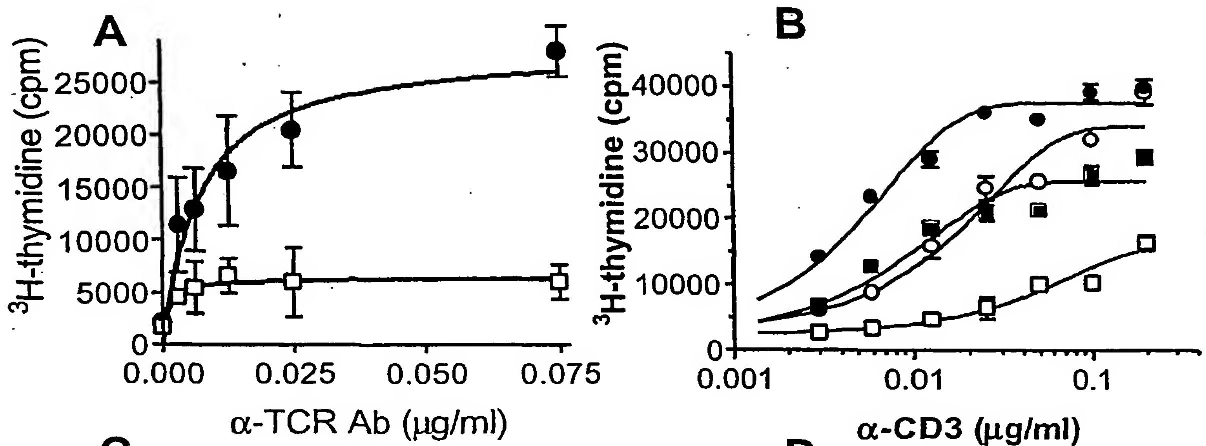
24. A method for treating or preventing an autoimmune disease in a subject comprising reducing T cell receptor clustering in the subject by increasing the amount of Mgat5 modified glycans, polylactosamine modified glycans, and/or lectin-Mgat5 modified glycan lattice on the surface of T cells of the subject.

25. A method of, and products for, diagnosing and monitoring conditions characterized by an abonormality in receptor clustering comprising assaying for differential glycosylation of the receptor.

26. A method as claimed in claim 25 wherein differential glycosylation is assayed by determining the presence of Mgat5 modified glycans, polylactosamine modified glycans, lectin-Mgat5 modified glycan lattice, or alterations in such glycans or lattice, compared to a control.

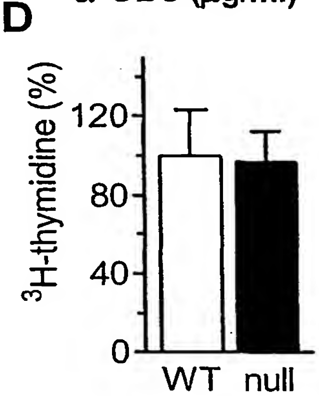
27. A method of, and products for, diagnosing and monitoring conditions characterized by an abnormality or defect of receptor clustering involving the interaction of a galectin and Mgat5 modified glycan or polylactosamine modified glycan comprising determining the presence of one or more complex as claimed in claim 1, a galectin-Mgat5 modified glycan lattice as claimed in claim 2, a Mgat5 modified glycan, a polylactosamine modified glycan, or one or more of an altered Mgat5 modified glycan, polylactosamine modified glycan, or a galectin-Mgat5 modified glycan lattice.

FIGURE 2



C

D50 and n_H values for TCR response			
Group	αCD28	D50 (ng/ml)	nH
-/-	+	4±0.5	59.6
-/-	-	11±1.8	33.8
+/+	+	12±0.8	16.7
+/+	-	~36	~6.0



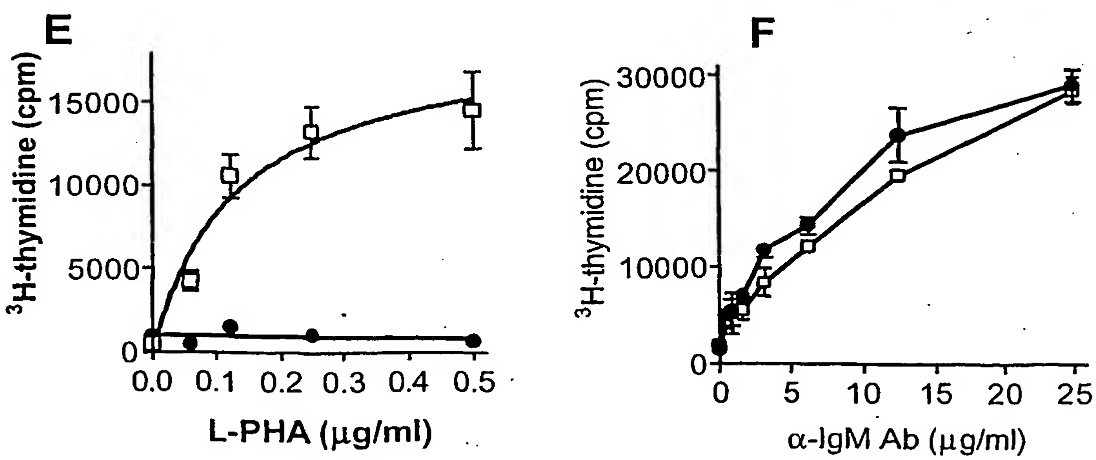
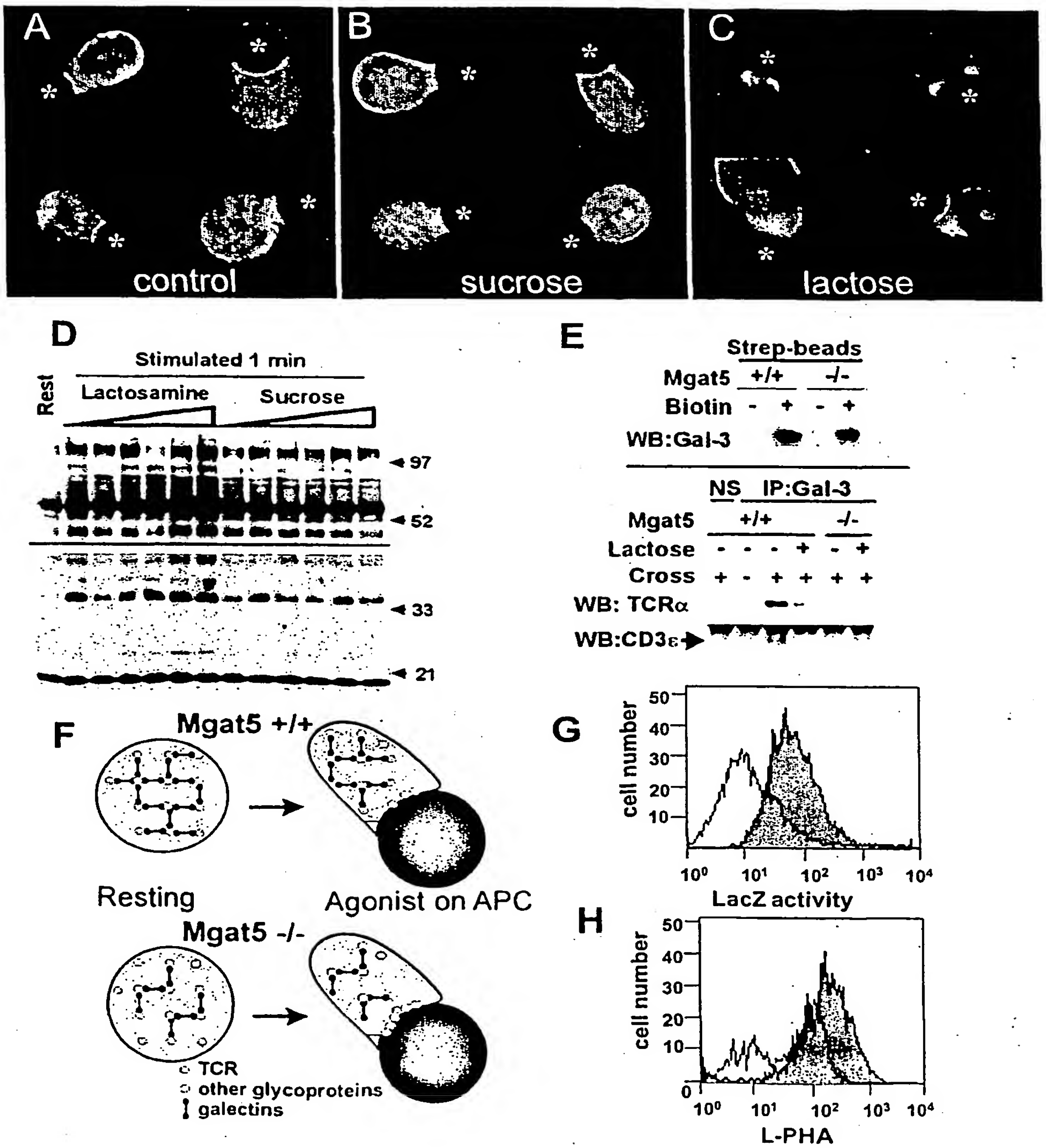


FIGURE 4


A
control
B
sucrose
C
lactose
D
Stimulated 1 min
Rest
Lactosamine
Sucrose
97
52
33
21
E
Strep-beads
Mgat5 +/+ -/-
Biotin - + - +
WB:Gal-3
NS IP:Gal-3
Mgat5 +/+ -/-
Lactose - - - + - +
Cross + - + + + +
WB: TCRα
WB:CD3ε
F
Mgat5 +/+
Resting
Agonist on APC
Mgat5 -/-
TCR
other glycoproteins
galectins
G
cell number
LacZ activity
H
cell number
L-PHA